# Early detection of diabetic kidney disease by urinary proteomics and subsequent intervention with spironolactone to delay progression: A prospective observational study with embedded randomised placebo-controlled trial

**Running title:** Urinary proteomics and spironolactone in type 2 diabetes

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## Summary

**Background:** Microalbuminuria is an early sign of kidney disease in diabetes and indicates cardiovascular risk. We tested if a prespecified urinary proteomic risk classifier (CKD273) was associated with development of microalbuminuria and if progression to microalbuminuria could be prevented with the mineralocorticoid receptor antagonist spironolactone.

**Methods**: Prospective multicentre study in people with type 2 diabetes, normal urinary albumin excretion and preserved renal function in 15 European specialist centres. High-risk individuals determined by CKD273 were randomised 1:1 (interactive web response system) in a double-blind randomised controlled trial comparing spironolactone 25 mg o.d. to placebo. Primary endpoint was development of confirmed microalbuminuria in all individuals with available data. Secondary endpoints included reduction in incidence of microalbuminuria with spironolactone and association between CKD273 and impaired renal function defined as a glomerular filtration rate < 60 ml/min per 1·73 $m^2$. This study is registered with ClinicalTrials.gov: NCT02040441 and is completed.

**Findings:** From March 25, 2014 to September 30, 2018 we followed 1775 participants, 12% (n=216) had high-risk urinary proteomic pattern of which 209 were included in the trial and assigned spironolactone (n=102) or placebo (n=107). Median follow-up time was 2·51 years (IQR 2·0-3·0). Progression to microalbuminuria was seen in 28·2% of high-risk and 8·9% of low-risk people (P< 0·001) (hazard ratio (HR), 2·48; 95% confidence interval [CI], 1·80 to 3·42 P<0·001, independent of baseline clinical characteristics). A 30% decline in eGFR from baseline was seen in 42 (19·4 %) high-risk participants compared to 62 (3·9 %) low-risk participants, HR 5·15; 95 % CI (3·41 to 7·76; p<0.0001). Development of microalbuminuria was seen in 35 (33%) randomised to placebo and 26 (25%) randomised to spironolactone treatment (HR 0·81, 95% CI, 0·49 to 1·34, P=0·41). Harms: hyperkalaemia was seen in 13 versus 4, and gynaecomastia in 3 versus 0 subjects on spironolactone and placebo, respectively.

**Interpretation:** In people with type 2 diabetes and normoalbuminuria, the urinary proteomic classifier CKD273 was associated with a 2·5 times increased risk for progression to microalbuminuria over a median of 2·5 years, independent of clinical characteristics. Spironolactone did not prevent progression to microalbuminuria in high-risk subjects.



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**Key words:** proteomics, diabetes, kidney disease, clinical trials, mineralocorticoid receptor antagonist

## Research in context

*Evidence before this study*

We searched PubMed from Jan 1, 1990 to Jun 30, 2019, for all English-language publications, using the search terms "type 2 diabetes", "normoalbuminuria", "urinary proteomics", "urinary peptidomics", "spironolactone", "mineralocorticoid receptor antagonist", "aldosterone antagonist", "albuminuria", "kidney disease", "nephropathy". Posthoc studies of cross sectional and longitudinal cohorts of persons with type 2 diabetes and non-diabetic kidney disease have been investigated with urinary proteomics as a marker of presence of kidney disease or a marker of development of kidney disease. The urinary peptide pattern for chronic kidney disease consisting of 273 peptides (CKD273) was demonstrated to be associated with progression of albuminuria or loss of renal function in such retrospective cohorts, but no studies were prospective or had the risk marker linked to a potential intervention. Mineralocorticoid receptor antagonists (MRA) have been demonstrated to lower urinary albumin excretion in short term studies of patients with moderate to severely elevated albuminuria, but long-term data are lacking, and studies aiming to prevent progression of normo- to microalbuminuria have not been done.

*Added value of this study*

To our knowledge, this paper describes the first prospective multicentre study evaluating the multi-dimensional CKD273 urinary proteomic classifier for risk stratification in individuals with normoalbuminuria and type 2 diabetes. This study demonstrates that CKD273 is effective as an early marker of risk for progression to persistent microalbuminuria in a prospective trial setting, and in addition is associated with development of impaired renal function.

This study tested whether the mineralocorticoid receptor antagonist spironolactone could delay or prevent development of confirmed microalbuminuria in subjects identified to be at high risk of progression based on the CKD273 proteomic biomarker. This was not demonstrated.

*Implications of all the available evidence*

Collectively, all the evidence suggests that the urinary proteomic based risk marker CKD273 is associated with early progression of diabetic kidney disease, with added value to the clinical characteristics being used today in the clinic including urinary albumin excretion and glomerular filtration rate. The early progression cannot be mitigated by treatment with the MRA spironolactone.

## Introduction

Diabetic kidney disease (DKD) is a frequent and costly complication of diabetes as well as a leading cause of renal failure. In addition, DKD is associated with a significantly increased burden of cardiovascular disease (CVD). Globally 1 in 11 adults has diabetes, and numbers are increasing according to the International Diabetes Federation Atlas from 2019. Despite an observed reduction in relative risk for end stage kidney disease (ESKD) in diabetes during the last decades the absolute number of people referred for ESKD treatment has more than doubled [1]. This likely results from the increasing prevalence of diabetes, combined with reduction in competing CVD mortality and increased eligibility for treatment of ESKD. The current situation mandates better prediction, prevention and treatment of DKD.

In clinical practice, DKD is diagnosed by albuminuria and/or decrease in estimated glomerular filtration rate (eGFR). Microalbuminuria is a marker of increased risk for CVD and ESKD.[2] Treatment of micro- and macroalbuminuria with renin-angiotensin aldosterone system (RAAS) blocking agents and control of cardiovascular risk factors has improved outcomes,[3] but the prognosis remains poor and many still progresses despite widespread prescribing of these agents as advocated by clinical guidelines. Recently studies have suggested pleiotropic and kidney protective effects of sodium-glucose cotransporter-2 (SGLT2) inhibitors and potentially glucagon-like peptiode-1 (GLP1) receptor agonists and they are now recommended by guidelines in type 2 diabetes (T2D) with established DKD[4].

Studies into prevention of microalbuminuria with angiotensin converting enzyme inhibitors (ACEi) or angiotensin II receptor blockers (ARBs) have shown conflicting results.[5,6] Biomarkers to identify people who stand to benefit most from preventative therapy would therefore be helpful. Depending on the pathophysiology underlying the biomarker this could also help guide intervention and precision medicine in DKD. Good et al. previously described a high dimensional urinary biomarker pattern composed of 273 peptides associated with overt kidney disease: CKD273.[7] The original studies included people with chronic kidney disease (CKD) on a mixed background. It has been possible to develop risk scores with the same methodology that are optimised for diagnosis of different kidney diseases, but the CKD273 has been robust across multiple causes of CKD including DKD. In retrospective studies this proteomic classifier identified subjects at risk for DKD

and progression in albuminuria class earlier than the indices currently used in clinical practice[8-10]. However, all data on CKD273 to date derive from *post hoc* analyses of previously conducted studies and analyses of stored samples.

Blocking the RAAS has been recommended in DKD, and it was suggested that more complete inhibition of the RAAS with mineralocorticoid receptor antagonists (MRA) like spironolactone added to RAAS inhibition could further improve renal protection [11,12]. A further reduction in albuminuria of approximately 20-30% was seen in short-term studies and anticipated to predict beneficial renal effects. Long-term data from phase 3 trials focused on endpoints such as ESKD are missing, as are studies using spironolactone to prevent the earlier stages of DKD. The components of CKD273 include collagen fragments and are assumed to relate to early fibrosis in the kidney. Therefore, spironolactone considered antifibrotic via blockade of aldosterone seemed a relevant intervention.

The "Proteomic prediction and renin angiotensin aldosterone system inhibition prevention of early diabetic nephropathy in type 2 diabetic participants with normoalbuminuria" (PRIORITY) study aimed to test the following: 1) to demonstrate that CKD273 is associated with development of persistent microalbuminuria in people with T2D and normoalbuminuria in a prospective study; 2) to determine whether intervention with a mineralocorticoid receptor antagonist (spironolactone) compared to placebo reduces the increased risk of developing microalbuminuria in people with a high-risk CKD273 pattern. Albuminuria is used as a biomarker in the clinic, in trials and also here as the endpoint for early progression of DKD, making it inheritably difficult for a new marker such as CKD273 to perform better than albuminuria. Thus, we also looked at changes in eGFR as secondary outcomes as well as potential harms.

# Methods

### *Study design and participants*

PRIORITY is an investigator-initiated, prospective, double-blind, randomised, placebo-controlled international multicentre clinical and observational study in people with T2D and normoalbuminuria funded by the European Commission's Seventh Framework programme. The detailed rationale, study design and methods for PRIORITY have been published elsewhere.[13] Each

partner was represented in the steering group, and the partner Hannover Clinical Trial Centre was responsible for data management and study monitoring.

The protocol and amendments were approved by the respective national competent authorities using in part the Voluntary Harmonisation Procedure and by the institutional local ethics committees (see supplementary material). The study was conducted in accordance with the International Conference on Harmonisation – Good clinical practice (ICH-GCP) and Declaration of Helsinki. Participants provided written informed consent. Pharmacovigilance was performed at Medizinsche Hochschule Hannover Germany and an external independent data monitoring committee (DMC) monitored safety (not efficacy) throughout the study based on data from HCTC analysed by the DMC statistician. Safety focus was on serious adverse events, decline in eGFR >30 and 40% from baseline, hyperkalaemia and gynecomastia. EU Clinical Trials Register (EudraCT: 2012-000452-34) and http://www.clinicaltrial.gov (NCT02040441) accessed Jan 5th 2020.

From March 25, 2014 to the end of inclusion on August 31, 2016, people aged 18-75 years with T2D, preserved kidney function and normoalbuminuria were included and followed till September 30, 2018. Main inclusion criteria were: normoalbuminuria (ratio of urine albumin [mg] to creatinine [g] (UACR) <30) in at least two out of three consecutive morning void urine samples and eGFR) >45 ml per minute per 1·73 $m^2$ of body-surface area at screening. Information on pre-study albuminuria was not collected. Main exclusion criteria were use of dual RAAS blockade or MRA, or heart failure requiring MRA. Participants were stratified into high- or low-risk groups based on their CKD273 score, of a single random spot urine sample collected at screening (for details see supplementary material). High-risk was defined as CKD273classifier score >0·154, low-risk as ≤ 0·154 as previously described.[9,13] All data are normalised to 28 collagen fragments in urine not affected by disease and variability is low [14].

***Randomisation and masking***

Participants in the high-risk group were randomly assigned in a double-blind fashion to either oral spironolactone 25 mg o.d. or matching placebo. Participants in the high-risk group were randomly assigned in a double-blind fashion stratified by centre and RAAS treatment (yes/no) (1:1 using an interactive web response system (see supplementary document for further details) to either spironolactone 25 mg o.d. or matching placebo using a computer generated randomisation scheme stratified based on use of RAAS blocking agents. There was no difference in appearance between

the medications for each treatment group. Medications for each treatment group were supplied in identical bottles labelled appropriately to maintain masking within the study. Participants and investigators were masked to group assignment. Subjects continued ongoing medication including RAAS inhibitors according to local standards of care. All participants in the low-risk group were followed without study intervention and continued treatment according to local guidelines.

***Procedures***

Participants with a high-risk proteomics pattern were provided with study medication after randomisation and seen for a safety visit at the study centre after 2 weeks, with local measurement of creatinine and potassium. Every 13th week, participants were seen in the clinic and provided with the study drug. At each visit, UACR was tested in three consecutive urine samples, and locally measured biochemistry was analysed. Standard of care treatment was encouraged for all.

Participants with a low-risk CKD273 pattern were seen once yearly after the baseline visit and tested for UACR in three consecutive urine samples as well as locally measured biochemistry.

Samples were analysed locally with standardised methods, as described previously.[15] eGFR was calculated centrally using CKD-EPI equation. UACR was measured at the central laboratory: Steno Diabetes Center Copenhagen, Denmark (SDCC) using Vitros® 5600 MicroSlide (Orto Clinical Diagnostics, New Jersey, USA).

Urine proteomics was performed by applying capillary electrophoresis mass spectrometry (CE-MS) analysis at the central laboratory at Mosaiques Diagnostics in Hannover, Germany. This provides data on >1000 identified proteins or peptides and a predefined renal risk profile based on 273 peptides (CKD273). [13,16] Originally a threshold was identified to separate subjects with CKD from healthy controls. Based on a post hoc analysis of CKD273 as a marker of progression from normoalbuminuria to microalbuminuria, we had a priori defined a threshold for CKD273 of >0·154 corresponding to the 20% percentile indicating high risk for progression from normo- to microalbuminuria .

***Outcomes***

The ***primary objective*** was to confirm that urinary proteomics is associated with development of confirmed microalbuminuria in people with T2D and normoalbuminuria. The primary endpoint was development of confirmed microalbuminuria in people with T2D and normoalbuminuria.

Confirmed microalbuminuria was defined as UACR>30 mg/g in at least two out of three first morning voids with 30% increase (geometric mean) in UACR from "run-in-phase" samples or >40 mg/g (geometric mean).

The ***secondary objective*** was to investigate if therapy with spironolactone 25 mg o.d. reduces risk of transition to microalbuminuria in those patients identified to be at high risk. ***Additional objectives*** were to compare the rates of change in UACR and eGFR in the high vs. low-risk groups, to compare the effect of spironolactone on rate of change in UACR in the intervention group and to study the association between the urinary proteomic patterns and renal events during the study including development of CKD 3 (for patients with eGFR>60 at baseline), slope of eGFR, and as a sensitivity measure >30 and 40% decline in eGFR from baseline or doubling of serum creatinine, based on one-time measurements of eGFR without requirement of confirmation by repeat testing.

***Safety outcome*** of special interest in the randomised high-risk population randomised in the intervention study included hyperkalaemia (plasma or serum level of potassium >0.4 mmol/L above local upper reference) and gynaecomastia. During hyperkalaemia the study medication was paused and could be restarted when potassium was normal. Adverse events and serious adverse events were recorded for the intention to treat study population and was not collected in the observational cohort (low risk patients).

***Statistical analysis***

Sample size for the primary objective of association between CKD273 status (high/low risk) and development of persistent microalbuminuria was smaller (n=333, see expanded statistical section in supplement) than for the secondary objective the effect of spironolactone vs. placebo in CKD273 high risk participants. So, in order to address the second objective more patients than needed for objective one was included. High-risk subjects were expected to comprise 15 % of the screened and included participants. Using the samples size formula for two proportions test ($\alpha = 0{\cdot}05$, $\beta = 0{\cdot}80$), randomised (1:1), 129 subjects in each arm of the intervention group would provide sufficient power to find 40% effect. We estimated that the study required 2000 subjects to be included in the observation cohort to accomplish this. The sample size was reduced by an amendment after a revised sample size calculation based on a review of the treatment effect of mineralocorticoid receptor antagonists[12]. Follow up time was also modified with the amendment (see details of amendments in supplementary material including the study protocol). Continuous variables are

reported as means with standard deviation (SD) for normally distributed data or median with interquartile range (IQR) for skewed data and are compared between groups using an unpaired t-test where skewed data are log transformed before comparison between groups. A chi-square test is used for comparison of categorical data. In the observation cohort for the primary objective, including all participants with valid proteomic score and data at baseline visit, a comparison between progression to persistent microalbuminuria in the high- and low-risk stratum was conducted using an unadjusted Cox-regression model with chi-square test. In addition, adjustment for age, sex, Hba1c, systolic blood pressure, retinopathy, eGFR and UACR at baseline was performed. For the secondary objective (effect of spironolactone in high-risk subjects), a comparison between spironolactone and placebo treatment was performed in the intention to treat cohort with a Cox-regression model including data on the primary outcome. The intention to treat cohort comprised all participants with a valid proteomic score with a high-risk pattern who were provided with study medication. To evaluate changes in UACR and eGFR over time a linear mixed model was applied and for UACR with adjustment for UACR at baseline followed by truncation to weeks in the study period. A two-tailed *p* value of <0·05 was considered significant. SAS Enterprise Guide version 7·1 (7.100.1.2711) (64-bit) by SAS Institute, Inc., Cary, NC, USA was used for statistical analysis.

### *Role of the funding source*

The study was overseen by an steering committee, not including members from the study funder. The funder had no role in the design of the study, the collection and analysis of the data, or writing of the report. All authors had access to the study results, and the first authors and corresponding author vouch for the accuracy and completeness of the data reported and had access to all data. The first authors and the steering committee had the final decision to submit for publication.

## Results

From March 25, 2014 through August 31, 2016, a total of 2277 persons from 15 study centres in 10 countries were screened and 1775 participants included. Of those, 216 participants were in the high-risk group and 1559 participants were in the low-risk group corresponding to a proportion of 12·3% high-risk participants. The main reason for screen failure was presence of microalbuminuria (n=133) (Figure 1). Baseline characteristics are presented in Table 1. In comparison to low-risk

individuals, the high-risk group were more likely to be male, and were older with longer diabetes duration, a lower eGFR and a higher UACR (P<0·02; Supplementary Table S1 and S2).

The trial ended with last study visit on September 30, 2018. The median follow-up time was 2·51 years (IQR 2·0-3·0). In the low risk group 150 subjects (9·6%) did not complete the follow-up period (Figure 1). Of the 216 high-risk participants 209 were randomised to either placebo or spironolactone. Median follow-up time in the intervention study was 2·5 years (IQR 2·0-3·1) and 36 participants (17 %) dropped out (Figure 1). During follow up there was more subjects in the high-risk group compared to low risk, who initiated treatment with glucose and blood pressure lowering medication, but no difference between the intention to treat groups (see table S6).

The primary endpoint of confirmed microalbuminuria was more frequent in high-risk individuals based on CKD273 where 61 of 216 (28·2%) developed the endpoint compared to 139 of 1559 (8·9%) low-risk individuals (p<0·0001) (Figure 2A). In a Cox-model the HR (high vs. low-risk) was 3·92**;** 95% confidence interval (CI), 2·90 to 5·30; p<0·0001. After adjustment for baseline age, sex, $HbA_{1c}$, systolic blood pressure, retinopathy, UACR and eGFR the HR was 2·48; 95% CI, 1·80 to 3·42; p<0·0001. Additional adjustment for glucose lowering or antihypertensive and diuretic medication at baseline or started during follow up, or for $HbA_{1c}$ during follow up, did not change the HR for CKD273 (Table S5+6). Endpoints given in Table 2.

In participants with eGFR>60 ml per minute per 1·73$m^2$ at baseline (n=1666), development of CKD stage 3 (eGFR<60 ml per minute per 1·73 $m^2$) was more frequent in high-risk individuals (n=48; 25·5%) compared to 119 (8·1%) low-risk individuals, (HR 3·93; 95% CI, 2·81 to 5·50) (Figure 2B). Few participants developed CKD stage 4 (eGFR<30 ml per minute per 1·73 $m^2$) during the trial period (7 (3·2%) in high-risk vs 3 (0·19%) in low-risk individuals (HR 16·70; 95% CI, 4·31 to 64·67). A decline in eGFR of 30% from baseline was seen in 42 (19·4 %) high-risk participants compared to 62 (3·9 %) low-risk participants, HR for CKD273 high-risk participants was 5·15; 95 % CI (3·41 to 7·76; p<0.0001) after adjustment for eGFR and UACR. A 40 % decline in eGFR from baseline was seen in 15 (6.9 %) high-risk participants compared to 22 (1.4 %) low-risk participants, HR 4·84; 95 % CI (2·43 to 9·68; p<0.0001) adjusted for eGFR and UACR. A doubling in serum creatinine from baseline was seen in 9 (4.17 %) high-risk vs. 9 (0.58 %) low-risk participants, HR 7·49, 95 % CI 2·97 to 18·90, p < 0·0001. No participants developed ESKD.

In agreement with this, we found a faster progression of albuminuria in high-risk participants, after adjustment for baseline UACR: 7·1 (SE 1·14) %/year, compared with low risk 2·6 (0·85) %/ year. Similarly, eGFR decline was faster in high-risk participants (Figure S3A). There was no difference in $HbA_{1c}$ or blood pressure during the study in high-risk vs. low-risk people (Figure S2). Adjusting for mean $HbA_{1c}$ during follow up or new antihypertensive or glucose lowering medication did not change the results.

For the secondary objective: effect of spironolactone in high-risk individuals according to CKD273 we found in the randomised study of spironolactone compared to placebo, that there was no significant difference in the development of the primary endpoint "confirmed microalbuminuria" between groups, as 35 of 107 (32·7%) placebo-treated individuals developed the endpoint compared to 26 (25·5%) of 102 individuals in the spironolactone group (HR 0·81; 95% CI, 0·49 to 1·34; p=0·41) (Figure 3 and S6). We had anticipated a 40% reduction in albuminuria progression, which cannot be excluded as it is within the 95% CI. Additional adjustment for glucose lowering or antihypertensive and diuretic medication started during follow up, or for $HbA_{1c}$ during follow up, did not change the HR for the intervention.

Development of CKD stage 3 (in subjects with baseline eGFR>60 ml per min per 1·73 $m^2$) was seen in 15 (16·7%) placebo-treated subjects and 33 (35·9%) spironolactone-treated subjects (hazard ratio 2·617; 95% CI, 1·42 to 4·82; Figure S5). There was no difference in change in eGFR over time in the two groups (Supplementary Figure S3B). A decline in eGFR of 30% occurred in 24 (23·5 %) participants allocated to spironolactone compared to 18 (16·8 %) in the placebo group, (HR for the spironolactone treated group was 1·61; 95 % CI (0·87 to 2·96; p=0·13). Few reached 40% decline.

Spironolactone-treated participants had similar $HbA_{1c}$ and blood pressure compared to placebo-treated participants (Supplementary Figure S4).

Adverse events are given for the intention to treat groups in tables 3, S4 and S5. Safety events of special interest, development of gynaecomastia resulted in discontinuation of study medication in 3 (3%) spironolactone-treated participants; and none in the placebo group. Hypotension led to discontinuation of study medication in a further 3 (3%) spironolactone-treated participants compared to 1 placebo-treated individual. Elevated serum potassium >5·5 mmol/L) occurred in 13 (13%) spironolactone-treated and 4 (4%) placebo-treated individuals.

## Discussion

To our knowledge, this is the first prospective trial using a proteomics-based signature for risk stratification followed by risk-based intervention. We found that in normoalbuminuric T2D individuals with preserved renal function higher CKD273 classifier scores were associated with increased risk for progression to confirmed microalbuminuria independent of clinical markers. The high-risk CKD273 pattern was also associated with decline in renal function evaluated as progression to CKD stage 3 and 4 or as decline in eGFR. This confirms our primary hypothesis that individual risk can be assessed early in the course of T2D based on urinary proteomics. Compared to placebo, treatment with the mineralocorticoid receptor antagonist spironolactone was not able to delay development of microalbuminuria or impaired renal function.

In current practice, confirmed microalbuminuria is used as a marker of onset of DKD and increased CVD risk although the underlying pathology may vary. In the presence of established microalbuminuria, RAAS blockade reduces progression to macroalbuminuria,[17] and multifactorial intervention targeting cardiovascular risk factors reduces renal and cardiovascular morbidity and mortality.[18] Although microalbuminuria is the earliest clinical index of renal damage, histological changes may already be advanced by the time it is detectable,[19]thus earlier identification of at-risk individuals is imperative in order to guide targeted preventive therapy.[20] Increases in urinary albumin to microalbuminuria or higher levels are strongly associated with progression to more serious clinical endpoints such as significant loss of renal function and eventually end stage kidney disease, but also to an increased risk for cardiovascular complications[21]. Furthermore, using progression to microalbuminuria as the endpoint is the only current option for a study of early intervention aiming to prevent or delay onset of DKD. In addition to the clinical utility, the CKD273 pattern could also be used for enrichment of future clinical studies, with a high-risk population for progression of albuminuria.

Previous *post hoc* analyses of cross sectional or longitudinal studies, collected without applying standardised protocols for collection, storage, transportation or analysis of samples, demonstrated that a high CKD273 score was associated with progression of renal disease in persons with and without diabetes.[10,22-24] Not all participants with elevated CKD273 risk score progressed to microalbuminuria within the trial with a median follow-up of 2·5 years. With longer follow-up more participants could potentially progress to microalbuminuria and the higher rate of drop out (17 vs. 10%) in the high-risk group could have led to a small underestimation of progression. Previous

studies have suggested that an elevation of the risk score precedes development of increased albuminuria with 3-5 years.[10] The increase in AUC of the ROC curve was statistically significant when adding CKD273 to the clinical variables. The clinical significance can be discussed, as the change in AUC was small (Fig S1). This may reflect that the AUC was already high (0·76) with the clinical variables alone and that AUC is a conservative measure of added value, which was supported by the statistically significant improvement in the discrimination index. It may also reflect that the CKD273 was not perfect with the current study design and duration. In 2672 subjects primarily diagnosed with diabetes (type 1 and 2) in which study rapid eGFR decline was the primary endpoint,[8] CKD273 had a stronger association with UACR in those with baseline eGFR>70 ml per min per 1·73 $m^2$, supporting the use of CKD273 in the present study population with preserved renal function. In the current trial only 12·3% of participants were classified as high-risk according to CKD273 score, which is less than the expected 15% based on a previous study cohort study of 700 individuals with T2D and normoalbuminuria.[9]

The results of the proteomic analyses were available within three days. This demonstrates feasibility of the test in a clinical setting. At present, urinary proteomic analysis is a high-end technology with costs significantly higher than urine albumin testing. The analysis currently costs 850 Euro/sample and the laboratory can do the analysis on shipped samples as in the trial and has a scalable platform, but currently the method cannot be set up in local laboratories. Health economic analyses previously indicated that use of CKD273 could be cost effective in T2D at this price. It requires that the CKD273 score is associated with development of microalbuminuria, and that progression can be prevented or delayed with preventive treatment.[25]The current study confirms the first of these criteria. In particular, it could be cost saving in people at high risk of CVD related complications. If CVD risk is low it is not cost effective, but most people with T2D are at elevated CVD risk. Regardless of the potential cost-effectiveness, restrictions on reimbursement in health systems may still be a limitation. Particularly in settings where screening with low cost methods including albuminuria and eGFR has not been implemented. Alternative use could be in clinical trials for selection of high-risk individuals or for evaluation of response to interventions.

Mineralocorticoid receptor antagonists have been shown to reduce albuminuria when added to ongoing RAAS blockade in subjects with diabetes and micro- or macroalbuminuria in short term trials of up to 1 year.[26,27] The compounds are expected to reduce fibrosis, and as CKD273 to a large extent is composed of peptides related to changes in extracellular matrix,[28] we expected

spironolactone to be effective in high-risk individuals. We did not demonstrate this, which could relate to study power, study duration or lack of effect in this population. It is a limitation that the number of high-risk participants randomised to intervention were lower than anticipated in the sample size calculation. The event rate in the placebo group was also lower than expected and thus power was reduced. Decline in eGFR over time was an additional secondary endpoint, which was similar in the two groups particularly if calculated from week 13 after the initial decline in eGFR (Table 2). In contrast more participants developed CKD stage 3 in the spironolactone groups compared to placebo, potentially due to acute hemodynamic effects (Figure S3B), and there was no significant increase in a 30% decline in eGFR from baseline. Treatment was well-tolerated. Alternative interventions which have recently demonstrated potential renal benefits in diabetes should be tested in high-risk individuals, such as non-steroidal mineralocorticoid receptor antagonists,[29]GLP1 receptor agonists or SGLT2 inhibitors[4].

Our trial has some limitations. The risk stratification to high- and low-risk was based on proteomic analysis of a single urine sample. We expect that the variation is limited due to the large number of peptides included in the pattern[7].This issue has not been extensively studied, but repeatability has been tested with 100% correct classification of CKD cases and controls on multiple occasions. In addition, the relative intraassay coefficient was 7%.[16] Microalbuminuria is an accepted clinically relevant surrogate for DKD, but not approved by regulatory agencies, although a recent conference with EMA and FDA discussed observational and clinical trial data showing a strong association between changes in albuminuria and long-term renal outcomes including ESKD.[30]Since the study was designed, relative changes in eGFR of 30 or 40% were suggested as outcome and high-risk based on CKD273 was strongly associated with these outcomes as well, although one-time measurements without repeated test for confirmation was used. The estimated hazard ratio for progression may be falsely low, as the high-risk group were treated with spironolactone or placebo, although this had no effect on albuminuria, but exclusion of subjects with high risk on active treatment did not change the hazard ratio (Supplementary Figure S6). Had spironolactone also been tested in the low risk group this could have increased the power but would also have exposed many without progression to the medication. As the low risk group was not randomised to spironolactone or placebo the trial cannot asses if spironolactone could be beneficial in this population or if screening and treatment is superior to non-screening, which would be particularly relevant if spironolactone had shown benefit. The major strengths of the study include a large, well-phenotyped cohort, prospective study design with up to 4·5 years follow-up. In the protocol

additional unblinded follow up was anticipated, but independent of the findings in the study, it has so far not been possible to fund further clinical follow-up. However, we are aiming for register based follow-up, where possible. The study was funded by an EU FP7 grant with time limits inherent in the grant which defined the stopping date, and without opportunity for funding of an extension. There was consistency in findings of CKD273 being associated with progression of albuminuria and loss of renal function.

In conclusion, the urinary proteomic classifier CKD273 is associated with a 2·5 times increased risk for progression to microalbuminuria and impaired renal function during a median of 2·5 years of follow-up, in subjects with T2D and normoalbuminuria, independent of clinical characteristics. Spironolactone did not prevent progression of albuminuria in high-risk subjects.

**Contributors:**

PR, HM, HvL, CD and MKL were involved in the design of the study. All authors were involved in the collection of data. PR, MKL and NT wrote the first draft of the report. MKL and NT contributed equally. All authors were involved in data analysis and interpretation, and in drafting and critically revising the report. All authors had access to study results and the first authors and corresponding author take responsibility for the integrity of the data and accuracy of the data reported. All authors reviewed and approved the final version of the report for submission.

**Conflict of interests:**

J.B. honoraria and lecture fees from Amgen, Boehringer Ingelheim, Nipro, M.L. has equity interest in Novo Nordisk A/S, A.L.B. reports having given lectures for Astra Zeneca, Novo Nordisk, Boehringer Ingelheim, Sanofi and has served on advisory boards for Astra Zeneca, Boehringer Ingelheim, Novo Nordisk, Sanofi, G.C. has given lectures and received meeting support from NAPP Pharmaceuticals Ltd.A.K. has received research grants from AstraZeneca, MSD and Novo Nordisk, all given to Bethesda Diabetes Research Center, A.O. reports having received research grants from Sanofi and Amgen and lecture fees from Sanofi, Amgen, Fresenius Medical Care, Amicus, and Kyowa-Kyrin and having served as a consultant for Freeline, Sanofi, and Amicus. F.P. reports having received research grants from AstraZeneca, Novo Nordisk, and Novartis and lecture fees from Novartis, Eli Lilly, MSD, AstraZeneca, Sanofi and Boehringer Ingelheim and having served as a consultant for Astra Zeneca, Bayer, Amgen, Novo Nordisk and MSD. J.R.P. fees from Novo Nordisk, non-financial support from Merck (Germany) and Itamar Medical, personal fees from Lilly, ACI Clinical, Pfizer, Astra Zeneca and grants from Janssen, MS has given lectures for Bayer, Boehringer Ingelheim, Siemens Healthineers, Takeda, has served as a consultant for Vifor Fresenius and has been a member of the advisory board of Otsuka. H.M. is the co-founder and co-owner of Mosaiques Diagnostics. M.N reports having received lecture fees from Eli Lilly, AstraZeneca, Sanofi, Novo Nordisk and Boehringer Ingelheim. , J.S. and P.Z. are employees of Mosaiques Diagnostics, PR reports having given lectures for Astra Zeneca, Bayer, Novo Nordisk and Boehringer Ingelheim, and has served as a consultant for AbbVie, Astra Zeneca, Bayer, Eli Lilly, Boehringer Ingelheim, Astellas, Gilead, Mundipharma, and Novo Nordisk, all fees given to

Steno Diabetes Center Copenhagen, and has equity interest in Novo Nordisk. S.B, J.W.J.B., C.D, L.F., P.G., R.G., T.H. G.D.L , M.F.-M., G.N., A.P., F.R.,I.R., P.L.R., G.S., M.T, N.T., and H.v.L., have no conflicts of interest

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**Data sharing:**

Individual de-identified participant data are not freely available because of the risk of patient re-identification but interested parties can request access to de-identified participant data or anonymised clinical study reports through submission of a request for access to the corresponding author provided the necessary data protection agency and ethical committee approvals are given in order to comply with current relevant legislation. The study protocol and statistical analysis plan are available as part of the supplementary material and at http://www.clinicaltrial.gov (NCT02040441).

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1

2

**Figure 1 Study outline**

**Figure 2 Progression to renal endpoints according to CKD273 status in the observational cohort**

Panel A shows high-risk (n=216) and low-risk (n=1559) type 2 diabetic subjects based on the urinary proteomic pattern CKD273 and their progression to confirmed microalbuminuria during follow up. After adjustment for baseline age, sex, $HbA_{1c}$, systolic blood pressure, retinopathy, UACR and eGFR the hazard ratio was 2·48; 95% CI, 1·80 to 3·42; P<0·0001. Panel B shows progression to CKD stage 3 (eGFR<60 ml per minute per 1·73 $m^2$) in participants with eGFR>60 ml per minute per 1·73 $m^2$ at baseline: high-risk (n=184) and low-risk (n=1423) .

**Figure 3 Effect of spironolactone on progression to renal endpoints in intention to treat population.** In high-risk type 2 diabetic subjects randomised to spironolactone (n=102) or placebo (n=107). Panel shows progression to confirmed microalbuminuria.

1

2 **Figure 1**

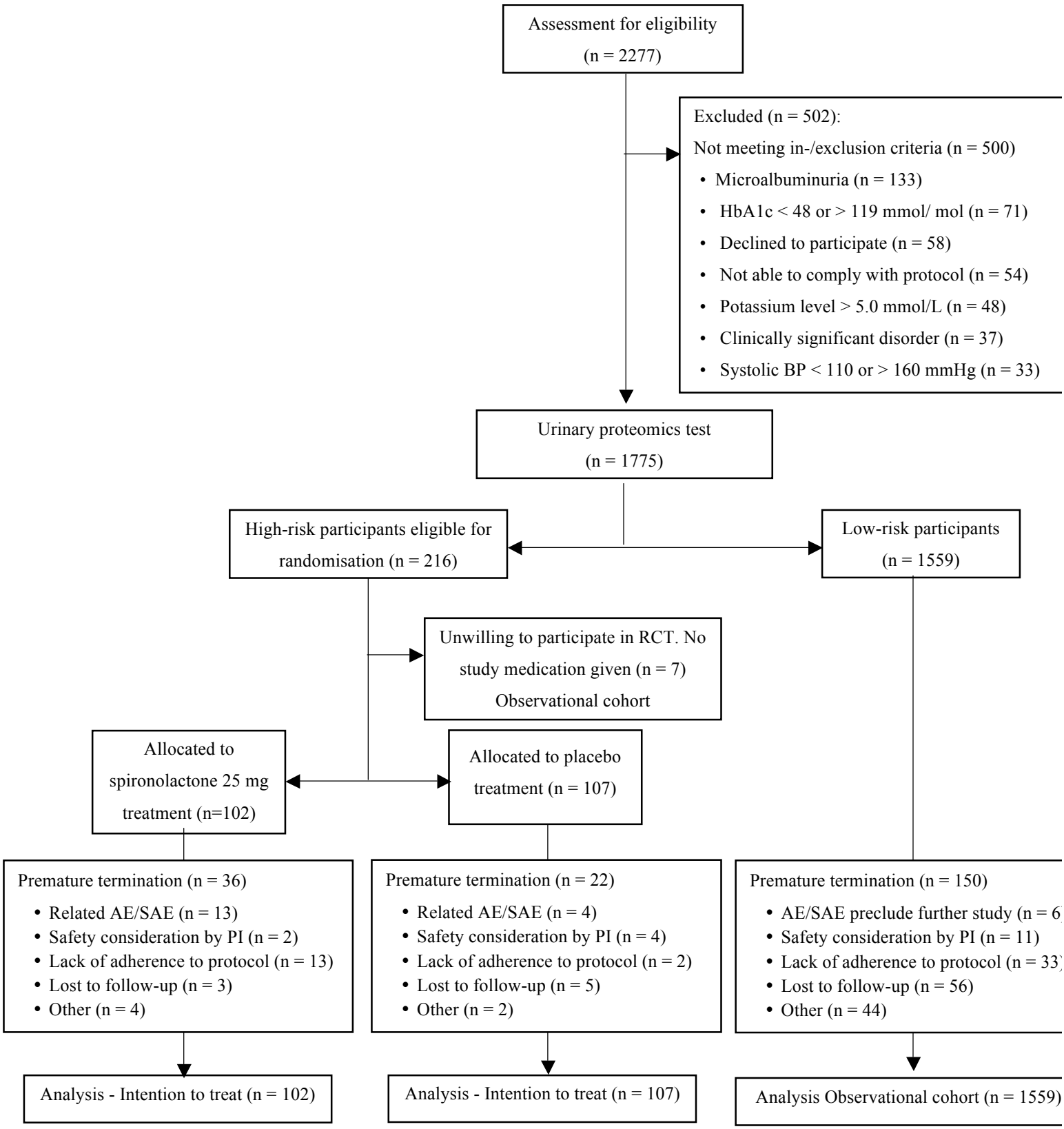
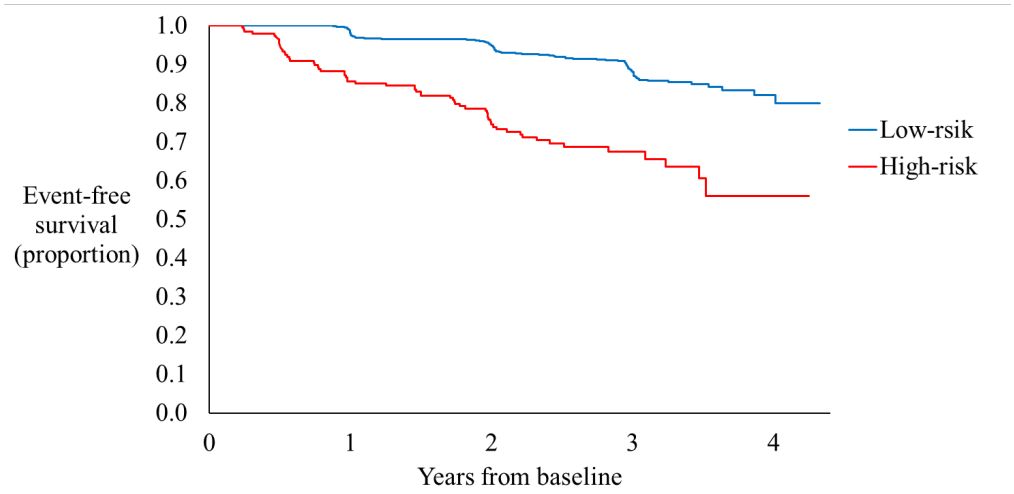
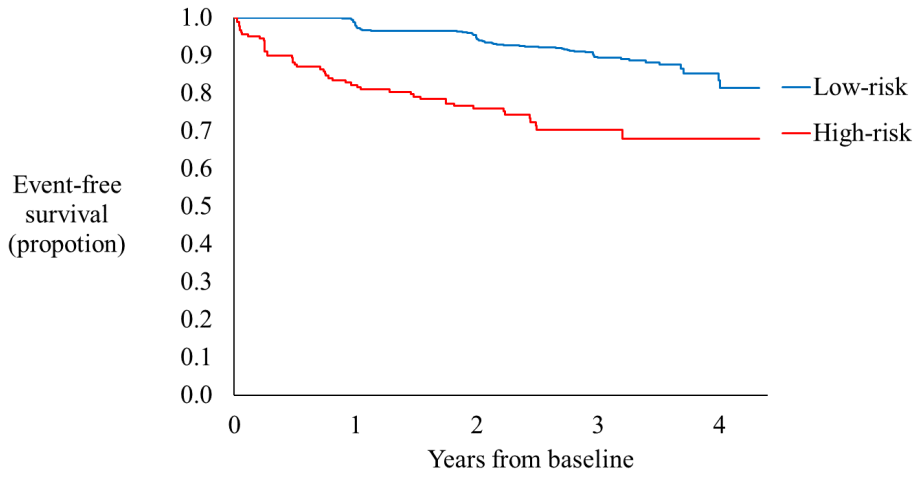

Assessment for eligibility
(n = 2277)
Excluded (n = 502):
Not meeting in-/exclusion criteria (n = 500)
• Microalbuminuria (n = 133)
• HbA1c < 48 or > 119 mmol/ mol (n = 71)
• Declined to participate (n = 58)
• Not able to comply with protocol (n = 54)
• Potassium level > 5.0 mmol/L (n = 48)
• Clinically significant disorder (n = 37)
• Systolic BP < 110 or > 160 mmHg (n = 33)
Urinary proteomics test
(n = 1775)
High-risk participants eligible for randomisation (n = 216)
Low-risk participants
(n = 1559)
Unwilling to participate in RCT. No study medication given (n = 7)
Observational cohort
Allocated to spironolactone 25 mg treatment (n=102)
Allocated to placebo treatment (n = 107)
Premature termination (n = 36)
• Related AE/SAE (n = 13)
• Safety consideration by PI (n = 2)
• Lack of adherence to protocol (n = 13)
• Lost to follow-up (n = 3)
• Other (n = 4)
Premature termination (n = 22)
• Related AE/SAE (n = 4)
• Safety consideration by PI (n = 4)
• Lack of adherence to protocol (n = 2)
• Lost to follow-up (n = 5)
• Other (n = 2)
Premature termination (n = 150)
• AE/SAE preclude further study (n = 6
• Safety consideration by PI (n = 11)
• Lack of adherence to protocol (n = 33
• Lost to follow-up (n = 56)
• Other (n = 44)
Analysis - Intention to treat (n = 102)
Analysis - Intention to treat (n = 107)
Analysis Observational cohort (n = 1559)

**Figure 2: Panel A Microalbuminuria observational cohort**



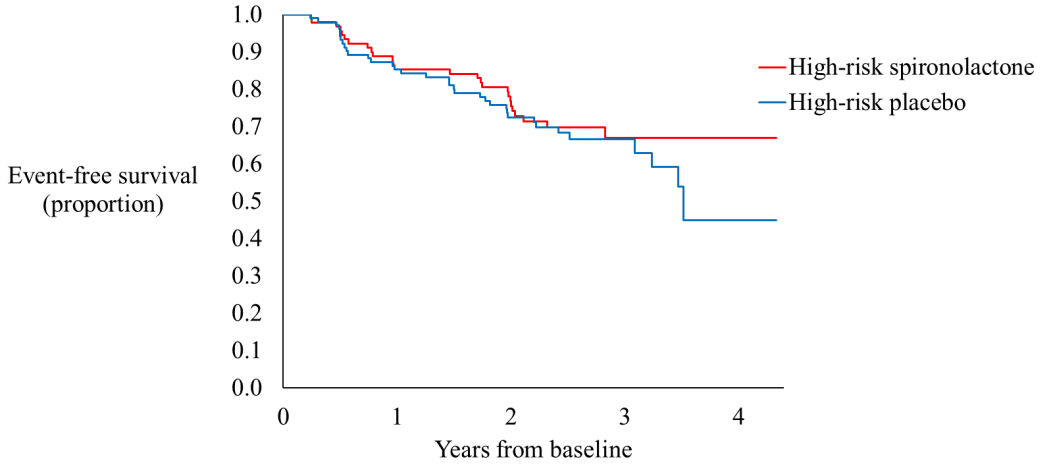

| No. of Participants | | | | | |
|---|---|---|---|---|---|
| High-risk | 216 | 189 | 157 | 63 | 13 |
| Low-risk | 1559 | 1482 | 1200 | 394 | 47 |

**Panel B Decline in renal function observational cohort**




| No. of Participants | | | | | |
|---|---|---|---|---|---|
| High-risk | 184 | 189 | 157 | 63 | 13 |
| Low-risk | 1423 | 1482 | 1200 | 394 | 47 |

**Figure 3 Microalbuminuria events in the high-risk intention to treat cohort**




| No. of Participants | | | | | |
|---|---|---|---|---|---|
| High-risk placebo | 102 | 98 | 79 | 33 | 6 |
| High-risk spironolactone | 107 | 86 | 74 | 29 | 7 |

**Table 1 Baseline characteristics by CKD273 subgroup and treatment**

| | Low-risk<br><br>n = 1559 | High-risk[a,b], Spironolactone<br><br>n = 102 | High-risk[a,b], Placebo<br><br>n = 107 |
|---|---|---|---|
| Male sex | 955 (61) | 69 (68) | 78 (73) |
| Age, years | 61 (9) | 63 (6) | 63 (7) |
| Known diabetes duration, years | 11 (8) | 14 (8) | 14 (9) |
| Body mass index, kg/ $m^2$ | 30 (5) | 30 (5) | 31 (6) |
| Systolic blood pressure, mmHg | 133 (12) | 135 (12) | 134 (12) |
| Diastolic blood pressure, mmHg | 78 (9) | 79 (9) | 79 (9) |
| eGFR, ml per minute per 1·73 $m^2$ | 88 (15) | 81 (15) | 82 (19) |
| UACR, mg/ g | 5 (3–8) | 7 (4–12) | 7 (4–12) |
| Potassium, mmol/ L | 4·2 (0·4) | 4·3 (0·5) | 4·2 (0·4) |
| Sodium, mmol/ L | 140 (3) | 139 (3) | 140 (3) |
| $HbA_{1c}$, mmol/ mol | 57 (12) | 58 (13) | 59 (13) |
| $HbA_{1c}$, % | 7·3 (1·1) | 7·5 (1·2) | 7·5 (1·2) |
| Total cholesterol, mmol/ L | 4·4 (1·0) | 4·4 (1·1) | 4·3 (1·1) |
| HDL cholesterol, mmol/ L | 1·2 (0·3) | 1·2 (0·3) | 1·2 (0·4) |
| LDL cholesterol, mmol/ L | 2·4 (0·9) | 2·4 (1·1) | 2·3 (1·0) |
| Triglycerides, mmol/ L | 1·6 (1·1-2·3) | 1·8 (1·2-2·6) | 1·7 (1·2–2·6) |

| | | | |
|---|---|---|---|
| CKD273, arbitrary units | -0·4 (0·3) | 0·4 (0·2) | 0·3 (0·2) |
| Smoking status | Current: 223 (14)<br>Never: 861 (55)<br>Former: 468 (30)<br>Unknown: 7 (< 1) | Current: 12 (12)<br>Never: 56 (55)<br>Former: 34 (33)<br>Unknown: 0 | Current: 8 (7)<br>Never: 56 (52)<br>Former: 43 (40)<br>Unknown: 0 |
| ACEi/ARB | 952 (61) | 90 (88) | 93 (87) |
| Follow-up time, years | 2·5 (2·0-3·0) | 2·5 (2·1-3·0) | 2·5 (2·0-3·1) |

Mean (SD) or median (IQR) for continuous variables, n (%, rounded) for categorical variables. Low-risk: CKD273 continuous-classifier below or equal to the cut-point of 0·154. High-risk: CKD273-classifier above the cut-point of 0·154. [a]Compared to baseline description [15] two participants were excluded in the high-risk group after inspection was not able to identify informed consent forms. [b]High-risk CKD273 was identified in 216 participants of whom 209 were randomized to intervention. See table S2 for baseline data in all 216. eGFR denotes estimated glomerular filtration rate, UACR Urine Albumin-to-Creatinine Ratio, ACEi angiotensin-converting-enzyme inhibitors and ARB angiotensin-II-receptor blockers

**Table 2 Primary and secondary outcomes in the observational cohort and in the intention to treat cohort.**

| **Observational cohort** | Low-risk<br>n = 1559 | High-risk<br>n = 216 | Hazard ratio (95% CI) | p value |
|---|---|---|---|---|
| Primary endpoint: Microalbuminuria confirmed, n (%) | 139 (8·9) | 61 (28·2) | 3·92 (2·90 to 5·30) | <0·0001 |
| Secondary endpoints | | | | |
| Microalbuminuria (single value), n (%) | 288 (18·5) | 99 (45·8) | 3·68 (2·93 to 4·62) | <0·0001 |
| Macroalbuminuria (confirmed), n (%) | 22 (1·4) | 2 (0·01) | 0·66 (0·15 to 2·81) | 0·57 |
| CKD stage3 (eGFR<60ml/min/1·73m$^2$)*, n (%) | 119 (7·6) | 48 (22·2) | 3·50 (2·50 to 4·90) | <0·0001 |
| Fatal and non-fatal cardiovascular outcome†, n (%) | 53 (3·4) | 12 (5·6) | 1·77 (0·92 to 3·22) | 0·089 |
| Ischaemic heart disease, n (%) | 24 (1·5) | 7 (3·2) | 2·22 (0·96 to 5.2) | 0·063 |
| Stroke, n (%) | 15 (0·96) | 4 (1·9) | 1·99 (0·66 to 6·0) | 0·22 |
| Congestive heart failure, n (%) | 8 (0·51) | 2 (0·93) | 1·96 (0·42) to 9·21) | 0·72 |
| All-cause mortality, n (%) | 11 (0·62) | 2 (0·93) | 1·41 (0·31 to 6·37) | 0·65 |
| Development of retinopathy or laser | 144 (9·2) | 21 (9·7) | 1·02 (0·65 to | 0·93 |

| | | | | |
|---|---|---|---|---|
| treatment (self-reported), n (%) | | | 1·62) | |
| Retinopathy, n (%) | 101 (6·5) | 14 (6·5) | 0·96 (0·55 to 1·68) | 0·89 |
| Laser treatment, n (%) | 54 (3·5) | 9 (4·2) | 1·21 (0·56 to 2·44) | 0.60 |
| | | | Difference (95% CI) | |
| Change in u-albumin /creatinine ratio (%/year)(SE) | 2·6 (0·85) | 7·1 (1·14) | 4·5 (2·7 to 6·2) | <0·0001 |
| Change in eGFR, ml/minute per 1·73 $m^2$ per year (SE) | 0·47 (0·19) | 1·37 (0·34) | 0·90 (0·14 to 1·67) | 0·206 |
| | | | | |
| **High risk randomised (Intention to treat cohort)** | High-risk Spironolactone n = 102 | High-risk Placebo n = 107 | Hazard ratio (95% CI) | p value |
| Primary endpoint: Microalbuminuria confirmed n (%) | 26 (26) | 35 (33) | 0·81(0·49 to 1·34) | 0·41 |
| Secondary endpoints | | | | |
| Microalbuminuria (single value), n (%) | 42 (41) | 57 (53) | 0·76 (0·51 to 1·14) | 0·18 |
| Macroalbuminuria (confirmed), n (%) | 0 | 2 (2) | 0·00 (0·00 to 5·59) | 0·52 |

| | | | | |
|---|---|---|---|---|
| CKD stage 3 (eGFR<60ml/min/1·73 $m^2$)*, n (%) | 33 (32) | 15 (14) | 2·88 (1·56 to 5·30) | 0·0007 |
| Fatal and non-fatal cardiovascular outcome†, n (%) | 4 (4) | 8 (7) | 0·57 (0·17 to 1·88) | 0·35 |
| Ischaemic heart disease, n (%) | 4 (4) | 3 (3) | 1·45 (0·33 to 6·7) | 0·60 |
| Stroke, n (%) | 0 (0) | 4 (3·7) | 0·00 (0·00 to 1·59) | 0·14 |
| Congestive heart failure, n (%) | 1 (1) | 1 (1) | 1·14 (0·071 to 18·2) | 0·93 |
| All-cause mortality, n (%) | 1 (1) | 1 (1) | 1·13 (0·071 to 18·1) | 0·93 |
| Development of retinopathy or laser treatment (self-reported), n (%) | 14 (14) | 4 (4) | 2·82 (1·08 to 7·4) | 0·034 |
| Retinopathy, n (%) | 9 (9) | 4 (4) | 2·71 (0·84 to 8.82) | 0.097 |
| Laser treatment, n (%) | 9 (9) | 2 (2) | 4.22 (0.88 to 20.3) | 0.073 |
| | | | Difference (95% CI) | |
| Change in urinary albumin/creatinine ratio %/year (SE) | 6·8 (2·5) | 6·4 (2·3) | 0·38 (-6·2 to 7·0) | 0·91 |
| Change in eGFR, ml/minute per 1·73 $m^2$ per year (SE) | -1·52 (0·54) | -1·33 (0·49) | 0·18 (-1·25 to 1·60) | 0·80 |

| | | | | |
|---|---|---|---|---|
| Change in eGFR from week 13,<br><br>ml/minute per 1·73 $m^2$ per year (SEM) | -1·33 (0·68) | -1·26 (0·64) | 0·073 (-1·8 to 2·0) | 0·94 |

*For patients with eGFR >60 ml/min/1·73m2 at baseline· Data are numbers (%) or mean (SE). SE denotes standard error of the mean.

†Comparison of composite fatal and non-fatal cardiovascular outcome (MI, stroke, CABG, PTCA, hospitalization for heart failure and CVD), and all-cause mortality during the study)

**Table 3 Adverse events in intention to treat cohort (high risk randomised to placebo or spironolactone).**

| | High-risk Spironolactone n = 102 | High-risk Placebo n = 107 |
|---|---|---|
| Any adverse events (total number) | 312 | 321 |
| Any adverse events (patients with at least one) | 82 (82) | 86 (80) |
| Adverse events leading to discontinuation of study drug | 25 (25) | 10 (9) |
| Any serious adverse event (patients with at least one) | 34 (33) | 22 (21) |
| Any serious adverse event | 17 (17) | 21 (20) |
| Serious adverse event considered related to study drug | 2 (2) | 1 (1) |
| Death | 1 (1) | 1 (1) |
| Events of special interest: | | |
| Hyperkalaemia | 9 (9) | 1 (1) |
| Event of s-potassium > 5·5 mmol/L | 13 (13) | 4 (4) |
| Gynecomastia | 3 (3) | 0 (0) |
| Hypotension | 3 (3) | 1 (1) |
| Development of CKD 3 (eGFR <60 ml/min/1·73m$^2$) | 33 (32) | 15 (14) |
| Development of CKD 4 (eGFR<30 ml/min/1·73m$^2$) | 3 (3) | 4 (4) |
| 30% decline in eGFR from baseline | 24 (24) | 18 (17) |

| | | |
|---|---|---|
| 40% decline in eGFR from baseline | 7 (7) | 8 (7) |

Data are numbers (%) of adverse events unless stated otherwise.